Amplification and Overexpression of Cyclin D1 in Breast Cancer Detected by Immunohistochemical Staining

Cheryl Gillett, Vera Fantl, Rosalind Smith, Charlotte Fisher, Jiri Bartek, Clive Dickson, Diana Barnes, and Gordon Peters

Imperial Cancer Research Fund, Clinical Oncology Unit, Guy's Hospital, London SE1 9RT, United Kingdom [C. G., C. F., D. B.]; ICRF Laboratories, London WC2A 3PX, United Kingdom [V. F., R. S., C. D., G. P.]; and the Fibiger Institute, Danish Cancer Society, DK-2100 Copenhagen, Denmark [J. B.]

ABSTRACT

Immunohistochemical staining with a monoclonal antibody against human cyclin D1 can be used to identify breast cancers that have an amplification of the q13 region of chromosome 11. In general, the intensity of staining is directly proportional to the degree of DNA amplification. In two unusual tumors, in which the CCND1 locus is highly amplified but staining is relatively weak, it appears that the DNA has undergone rearrangement and that the amplified/rearranged CCND1 allele may have reduced transcriptional activity. More significantly, the immunohistochemical technique identifies additional tumors in which the cyclin D1 gene is overexpressed with only marginal or undetectable increases in copy number, implying that other mechanisms can lead to deregulated expression. These results suggest that the frequency of overexpression is much higher than previously concluded from DNA-based analyses and that more than one-third of human breast cancers may contain excessive levels of cyclin D1. The technique we describe should facilitate the detection of this abnormality in a clinical setting and clarify its prognostic significance.

INTRODUCTION

The loss, rearrangement, and amplification of particular regions of the genome are common features in human cancers, and since they are likely to influence the behavior of the tumor cells there is considerable interest in identifying the genes on which these chromosomal abnormalities impinge. Approximately one in six primary human breast cancers show amplification of genomic DNA centered on band q13 on the long arm of chromosome 11 (reviewed in Refs. 1 and 2). In a comprehensive survey of 183 patients who attended the Imperial Cancer Research Fund Breast Cancer Unit at Guy’s Hospital (3), we previously showed that this chromosomal abnormality is primarily associated with tumors classified as positive for estrogen receptor (>20 fmol/mg protein). Similar correlations have been reported by other groups, and there are preliminary indications that the presence of CCND1 is associated with tumors classified as positive for estrogen receptor.

Amplification of the q13 region was originally detected by Southern blotting of tumor DNA using probes for FGF3 (INT2) and FGF4 (HST1), two established oncogenes, and BCL1, a nontranscribed region affected by the t(11;14) translocation in centrocytic lymphoma and other B-cell neoplasms (1, 2). However, using RNA protection assays, we showed that FGF3 and FGF4 are generally not expressed in human breast cancers irrespective of the gene copy number (3). Despite sporadic reports of RNA transcripts, mainly based on in situ hybridization techniques, most of the human tumors and tumor cell lines analyzed do not express these oncogenes at detectable levels (18, 22–25). As a result, it is now accepted that FGF3 and FGF4 are unlikely to contribute directly to mammary tumorigenesis in humans and that the selective force for amplification must be provided by another gene or genes on the amplified DNA.

Among the candidates for this gene is CCND1, also referred to as D11S287 or PRADI, which we previously showed is located close to the BCL1 breakpoint and is consistently coamplified with FGF3 and FGF4 (26, 27). Indeed, it is now clear that CCND1 is activated by the t(11;14) translocation in B-cell neoplasms, as a result of juxtaposition with regulatory elements from the immunoglobulin heavy chain locus, as well as by intrachromosomal inversion in parathyroid adenomas, where it comes under the influence of the parathyroid hormone gene on 11p15, hence the name PRADI (28–32). Such observations imply that deregulated expression of CCND1 is contributing directly to the phenotype of these lesions and that it therefore qualifies as a proto-oncogene.

In terms of breast cancer, the most persuasive evidence implicating CCND1 is that it is expressed at elevated levels as a consequence of the 11q13 amplification (26). In the original report, we showed that this was true in both primary tumors and cell lines derived from breast and squamous cell carcinomas (26). Moreover, the gene was independently cloned by virtue of its overexpression in such cell lines (25). Other groups have subsequently confirmed these findings in both mammary and esophageal tumors (22, 33–35). However, it has only recently become possible to investigate the distribution of the cyclin D1 protein in tumor sections (36).

Cyclin D1 belongs to a family of proteins that functions primarily in the cell division cycle by regulating the activity of cyclin-dependent protein kinases (37, 38). Thus, cyclin D1 cDNAs were independently isolated by their ability to complement for G1 cyclin function in yeast and by their accumulation in the G1 phase of the cell cycle upon stimulation of growth-arrested macrophages with a specific cytokine (39–41). However, the exact roles of cyclin D1 and its close relatives, cyclins D2 and D3, remain enigmatic since all three appear to associate with the same kinase partners (42–44), and most breast cancer cell lines express more than one D-type cyclin (34, 35, and data not shown). Here we describe the use of a cyclin D1-specific antibody to stain breast cancer sections that are known to have amplification of the CCND1 locus. As well as discriminating amplified from nonamplified cases, the immunohistochemical procedure we describe can identify additional tumors in which the gene is overexpressed without an apparent increase in copy number. Staining with the monoclonal antibody therefore provides a more rapid assay for the amplification of chromosome 11q13 and a more accurate indication of deregulated expression of cyclin D1.

MATERIALS AND METHODS

Tumor Material. The tumors analyzed in this study formed part of a previous survey of 183 cases that had been assessed for amplification of FGF3 and CCND1 (3, 26). All were from women who had primary breast carcinoma between 1987 and 1988, and the clinicopathological profiles of these patients were described in detail previously (3). Of the 63 cases examined here, 20 were deliberately selected because of previous evidence for amplification of markers on chromosome 11q13, but no other selective criteria were applied. The other
43 cases were chosen at random from the residual pool of nonamplified tumors.

**Immunohistochemical Staining of Tumor Sections.** The primary antibody used for staining of sections was an IgG2a monoclonal antibody prepared against recombinant human cyclin D1 (45). In immunoprecipitation and immunoblotting experiments, the DCS-6 antibody was shown to be specific for cyclin D1 and did not cross-react with either cyclin D2 or cyclin D3 (45).

Blocks of primary tumor were fixed in either heated formal calcium or unheated methacarn, the two fixatives in routine use at the time. Preliminary evaluation showed that methacarn-fixed material gave superior nuclear preservation and stronger staining, and the method was optimized accordingly. Sections (3 μm) of methacarn-fixed tissues were cut and air dried on poly-l-lysine-coated slides. Dewaxed and rehydrated sections were preheated in distilled water at 37°C, treated for 4 min with 0.02% Pronase E (Sigma P9611) in PBS2 at 37°C, and rinsed in ice-cold water and PBS. All subsequent incubation steps were performed at room temperature, and slides were rinsed thoroughly with PBS between each step. After the sections were blocked in 20% fetal calf serum in PBS for 10 min, excess serum was removed and sections were incubated overnight with a 1:1000 dilution of ascites fluid containing DCS-6. For each case, a corresponding section was incubated in PBS without the primary antibody to control for nonspecific staining. Sections were then incubated for 30 min with biotinylated rabbit anti-mouse immunoglobulin (Dako) diluted 1:400 in PBS containing 0.3% normal human serum containing DCS-6. For each case, a corresponding section was incubated in PBS without the primary antibody to control for nonspecific staining. Sections were then incubated for 30 min with biotinylated rabbit anti-mouse immunoglobulin (Dako) diluted 1:400 in PBS containing 0.3% normal human serum and 1.5% fetal calf serum. Preformed peroxidase-conjugated streptavidin-biotin complex (Dako) was applied for 30 min, and peroxidase activity was demonstrated by applying 0.05% diaminobenxidine containing 0.6% hydrogen peroxide for 10 min. After the samples were rinsed thoroughly in PBS and then water, nuclei were counterstained in hematoxylin, and sections were dehydrated, cleared, and mounted.

**Staining Assessment.** Demonstration of cyclin D1 was assessed according to the intensity of the majority of stained cells. Hence, four categories of staining were established: weak, moderate, strong, and very strong.

**Southern Blot Analyses of Tumor DNA.** As described in previous reports (3, 26), total cellular DNA was isolated from primary breast tumor tissue using an Applied Biosystems 340A DNA extractor. Between 0.3 and 0.5 g of tissue were frozen in liquid nitrogen and disaggregated using a Braun Mikro-dismembrator II prior to extraction. Samples (15 μg) were digested with the restriction enzyme PstI and fractionated by electrophoresis in 0.8% agarose gels. Gels were treated with 0.25 M HCl for 10 min, and the DNA was transferred to Hybond N+ membranes (Amersham International) using 0.4 M NaOH. Hybridization conditions were based on those described by Church and Gilbert (46), and the DNA fragments used as probes in these analyses have been described in detail elsewhere (3, 25–27).

**Analysis of Tumor RNA.** RNA was prepared from primary tumor tissue by homogenization in guanidinium thiocyanate and centrifugation through cesium chloride (47). Samples (5–10 μg) were denatured in the presence of formaldehyde, fractionated in agarose gels, and transferred to Hybond N membranes. The RNA was immobilized by irradiation with UV light and hybridized to 32P-labeled DNA probes (46). Transcripts from the cyclin D1 gene were detected with a probe corresponding to the coding domain (generated by polymerase chain reaction), and blots were subsequently rehybridized with the 36B4 probe against the human acidic ribosomal protein PO (48).

**RESULTS**

**Immunohistochemical Detection of Cyclin D1.** In an initial feasibility study, aimed at detecting cyclin D1 by immunohistochemistry, we compared a variety of different protocols and antibodies. The main criteria were that staining should be definitive, reproducible, and capable of discriminating different levels of expression, for example, in tumors in which the CCND1 gene is modestly or highly amplified. In this regard, the DCS-6 monoclonal antibody against bacterially expressed cyclin D1 (45) was found to be more reliable than polyclonal antiserum against synthetic peptides (44). Similarly, methacarn-fixed material yielded superior nuclear preservation and stronger staining than material fixed in formal calcium, the other fixative used in the laboratory when the tissue samples analyzed in this study were taken. More recently, we have invested considerable effort in achieving satisfactory results with other more commonly used fixatives such as formal saline and phenol formal saline (49). In all cases, it is necessary to preincubate sections with proteases to reveal the antigen, and conditions were optimized by comparing treatments with trypsin, chymotrypsin, Pronase E, or protease XXIV for various times. As an alternative, preliminary data show that microwave treatment can be used to expose the antigen in routine, formalin-fixed material while retaining good morphological preservation.

The optimized protocol described in “Materials and Methods” was applied to a total of 63 methacarn-fixed breast carcinomas. Samples of benign and normal breast tissue were also stained to ascertain the level and incidence of cyclin D1 expression in nonmalignant lesions. Staining was predominantly but not exclusively nuclear, and tumor cells showed a range of staining intensities (e.g., Fig. 1, a–d). In some cases with strong nuclear staining, the cytoplasm was also stained, but it was rare to find cytoplasmic staining without nuclear staining and mitotic figures were not stained. Although there could be considerable variation within an individual tumor (see Fig. 1e for example), it was relatively straightforward to rank the degree of staining based on the overall appearance of the sections, and different observers generally reached the same conclusions. Sections of benign and normal tissues and areas of normal breast epithelium within a tumor were consistently scored as weakly staining (Fig. 1e). Of the 63 tumor sections analyzed in the study, 36 were considered to have a “normal” (i.e., weak) staining intensity, whereas 8 were classed as having moderate, 11 as strong, and 8 as very strong staining.

**Correlation of Cyclin D1 Staining and DNA Amplification.** The tumors selected for analysis had been previously tested for amplification of the CCND1 gene (26). With one exception (see below), all of the tumors reported to have amplified DNA showed moderate to very strong staining with the cyclin D1 antibody (Table 1). In the majority of cases, the intensity of staining and the degree of DNA amplification were in close agreement, but there were also some anomalies. For example, eight tumors that stained significantly above normal had not been classified as having an 11q13 amplification in earlier reports (Table 1). We therefore decided to reassess the amplification status of CCND1 in these anomalous cases.

Estimates of DNA copy number were obtained by comparing the autoradiographic signal from the target gene on 11q13, in this case CCND1, with that of a control marker, CD3y, on a nonamplified region of chromosome 11 (Fig. 2). On this basis, 8 of the tumors shown in Fig. 2 were previously reported to have amplification of 11q13 (26). All of these showed above normal staining with the monoclonal antibody and tumors 364 (moderate), 377 (strong), and 144 (very strong) are included in Fig. 1 to illustrate the different staining categories. At the other end of the scale, tumors such as 399 and 423 were deemed to have a normal DNA copy number, consistent with weak immunohistochemical staining with the antibody. An apparent discrepancy arises, however, with a tumor such as 382 which showed no apparent amplification at 11q13 (Fig. 2) despite strong staining.

<table>
<thead>
<tr>
<th>Staining</th>
<th>Amplified</th>
<th>Not amplified</th>
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<tbody>
<tr>
<td>Very strong/strong/moderate</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>Weak</td>
<td>1</td>
<td>35</td>
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*The abbreviation used is: PBS, phosphate-buffered saline.*
staining with the antibody (not shown). On the other hand, although
tumors 394 and 404 were previously considered borderline examples
of amplification (and therefore scored negative), their significant
staining with the antibody (Fig. 1e) would be consistent with the
modest increase in DNA copy number apparent in Fig. 2.

Correlation of Cyclin D1 Staining and RNA Expression. Since
the overexpression of cyclin D1 could be brought about by mecha-
nisms other than DNA amplification, we next asked whether the
staining of tumors such as 382 reflected increased transcription of the
CCND1 gene. Samples of total RNA, when available, were analyzed
on Northern blots hybridized with a probe for human cyclin D1 (Fig.
3). As previously reported (26), all human breast tumors appear to
express detectable levels of the major 4.3-kilobase cyclin D1 tran-
script, irrespective of amplification. By comparison with a control
probe, in this case the ribosomal gene 36B4 (48), cyclin D1 transcripts
were consistently more abundant in tumors that showed above normal

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Fig. 1. Immunohistochemical staining of cyclin D1 in primary breast tumors. a, weak uniform staining of a tumor with no detectable amplification of CCND1; b, moderate staining of tumor but not stromal cells (T364); c, strong staining (T377); d, very strong staining (T144); e, weak staining of normal breast and strong staining of tumor cells in the same section (T404); f, moderate staining of the highly amplified tumor, T123. All photographs were taken at $\times 400$ magnification.
staining for the gene product. Tumors 364, 377, and 144 again serve as typical examples of moderate, strong, and very strong expression (compare Figs. 1 and 3), whereas the nonamplified tumors, 399 and 423, expressed lower, presumably normal, levels of RNA, consistent with weak immunohistochemical staining with the antibody. The more ambiguous cases in terms of DNA amplification, 394 and 404, clearly had elevated levels of cyclin D1 RNA, but tumor 382, which showed an apparently normal DNA copy number, also expressed near-normal levels of the RNA (Fig. 3). Although some amplified and strongly staining tumors showed relatively low levels of cyclin D1 RNA, we attribute this to partial degradation of the RNA during the storage and extraction of the biopsy material.

**Tumors with Anomalous Staining Intensities.** The data summarized in Table 1 identified one example (tumor 97) in which the cells stained weakly with DCS-6 despite previous evidence for amplification of CCND1. Similarly, tumor 123 ranked among the most highly amplified at the DNA level but showed only moderate immunohistochemical staining (Figs. 1f and 2). Further analysis of the DNA provided a potential explanation. In tumor 97, a novel restriction fragment was observed with a probe for CCND1, indicating that the locus had undergone rearrangement as well as amplification (Fig. 4).

By cloning such a novel fragment, we have established that the rearrangement involves an as yet unidentified locus that maps more distally on chromosome 11q (not shown). Significantly, it is the rearranged allele that has undergone amplification, along with other markers in the region, such as FGF3 and FGF4, while the normal CCND1 allele appears unaffected or only weakly amplified (Fig. 4; Ref. 2). The relatively weak immunohistochemical staining implies, therefore, that the highly amplified allele is not expressed. It was not possible to analyze the RNA from this tumor because of lack of material. In tumor 123, a similar situation may prevail since the degree of DNA amplification of BCL1 is considerably less than that of markers that map on its centromeric (D11S146) and telomeric flanks (CCND1 and FGF3) on chromosome 11 (Fig. 4; Refs. 3 and 26). Both the RNA levels (Fig. 3) and the staining intensity (Fig. 1f) correlate more closely with the reduced amplification detected with the BCL1 probe, again suggesting that some of the amplified DNA is transcriptionally inactive.

**DISCUSSION**

Since the amplification of chromosome 11q13 is only observed in specific cancers and affects a localized cluster of genes, it is generally assumed that excessive expression of one or more of these genes is selected for during tumorigenesis. Implicit in this assumption is that the amplification contributes in some way to the tumor cell phenotype and may therefore have a significant bearing on the clinical outcome of the disease. For example, there are already indications that the presence of the 11q13 amplicon correlates with an adverse prognosis (4, 6, 7, 9, 10, 12), but the numbers analyzed are generally too low and the duration of the studies too short to reach definitive conclusions. Therefore, one of the aims of the current work was to facilitate the detection of the 11q13 amplicon and open up the possibility of analyzing larger groups of patients as well as archival material.

To this end, we chose to examine the levels of cyclin D1 since it is one of the few genes on the amplified DNA that are known to be expressed in mammary tumors and one of the most consistently amplified (2, 25, 26). In the majority of tumors, there was a clear correlation between the intensity of staining with the cyclin D1 antibody, the level of CCND1 RNA expression, and the degree of DNA amplification. Although inherently subjective, the immunohistochemical protocol we describe identified every known example of 11q13 amplification among the tumors surveyed, as well as additional cases in which the increase in DNA copy number was considered marginal. Assessing modest increases in DNA copy number can be difficult since the results can be influenced by the size of the DNA fragment, the specific activity of the probe, the efficiency/uniformity of DNA transfer, and the nonlinearity of the autoradiographic signal.
While it might be possible to improve the speed and discrimination of such methods by using phosphorimaging rather than autoradiography or by developing polymerase chain reaction-based procedures (50), all methods based on extracting total nucleic acids or protein from a tumor biopsy specimen can only provide a tissue-averaged figure. In contrast, immunohistochemical staining reveals the levels of protein within individual tumor cells. The heterogeneous staining of tumors such as 404 (Fig. 1e) illustrates how the extent and frequency of DNA amplification may have been underestimated in previous reports due to the presence of normal cells in the biopsy.

Perhaps the most significant advantage of immunohistochemical staining is that it has revealed cases in which there is elevated expression of cyclin D1 in the absence of any measurable change in the DNA copy number. If the cases analyzed thus far are assumed to be representative, then the deregulated expression of cyclin D1 may be twice as frequent as initially thought from DNA analyses. Thus, in such methods by using phosphorimaging rather than autoradiography, while it might be possible to improve the speed and discrimination of such methods, the anomaly tumors 97 and 123 appear to be important. For example, the stability of cyclin D1 might be affected by mutations in the protein itself, or in a regulatory kinase or phosphatase, and we are currently investigating such possibilities.

Whatever the mechanisms, the fact that cyclin D1 can be overexpressed in different ways suggests that it plays a significant role in tumorigenesis and is not simply a passenger on the amplified DNA. On the other hand, the anomalous tumors 97 and 123 appear to undermine this argument since they show only modest expression of cyclin D1 despite massive amplification of the DNA. In tumor 97, the most likely explanation is that rearrangement of the amplified DNA, which occurs very close to the 5' end of the CCND1 gene (Fig. 4 and data not shown), has affected transcription from the amplified allele. In tumor 123, the situation is less clear since we have not observed a rearrangement with the available DNA probes. However, since the BCL1 probe gave a much weaker signal than the two flanking markers, D11S146 and CCND1, there has presumably been some loss or disruption of the intervening DNA.

Several studies have described tumors in which adjacent markers are amplified to different extents or in which the degree of amplification does not correlate with the relative locations of these markers in the genome (52-56). One interpretation for such findings is that there may be more than one gene in the 11q13 region that can provide a selective focus for amplification. For example, the EMS1 gene which maps approximately 800 kilobases telomeric of CCND1 (Fig. 4a; Ref. 57) is also overexpressed as a result of DNA amplification (25, 58). EMS1, FGF3, and CCND1 are almost always coamplified (see tumor 780 in Fig. 4), and it could be argued that EMS1 is a better candidate for the key gene in tumors 97 and 123 since it has not been subject to rearrangement (Fig. 4). Moreover, in tumor 123 the over-expression of EMS1 is more dramatic than that of CCND1 (2).

However, tumor 123 is highly unusual, since it also expresses FGF3 (3), making it difficult to establish which of these genes is having the most significant influence on tumor phenotype. Conversely, there are clearly tumors in which EMS1 is excluded from the amplicon and elevated expression of cyclin D1 can occur without a concomitant increase in EMS1 RNA (2).

An alternative explanation for the anomalous tumors is that relatively modest increases or simply deregulated expression of cyclin D1 may be sufficient to achieve a biological effect. Indeed, it could be argued that too much cyclin D1 may be toxic to the cell. For example, overexpression of a classical mitotic cyclin leads to cell cycle arrest since the protein has to be degraded for cells to complete the cycle (59). Similarly, attempts to overexpress cyclin D1 in rodent fibroblasts have been complicated by reduced viability of the transfected cells (60). This paradox could of course be resolved if tumor cells had sustained additional mutations that allowed them to tolerate the over-expression of cyclin D1 or if the amplified DNA had undergone rearrangements that moderated the level of expression. Whatever the explanation, it is clear that D1 is not alone among the cyclin family in being implicated in tumorigenesis. As well as sporadic reports of increased levels of cyclins D2 and E in colorectal and mammary tumors (34, 35, 61), cyclin D2 is a target for murine leukemia virus insertion in mouse thymomas (62) and cyclin A has been disrupted by hepatitis B virus in a human hepatoma (63).

In the short term, it is not absolutely necessary to establish whether cyclin D1 is the key or only oncogene on the 11q13 amplicon since it can clearly provide a rapid, accurate, and practical assay for the vast majority of the tumors that show this abnormality. In the longer term, we hope that increased knowledge of the function of this gene may not only refine the diagnosis of particular categories of breast cancer but lead to novel therapeutic strategies.

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